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Fungicidal Action of Aureobasidin A, a Cyclic Depsipeptide Antifungal Antibiotic, against *Saccharomyces cerevisiae*

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Aureobasidin A, an antifungal antibiotic inhibiting a wide range of pathogenic fungi, is lethal for growing cells of susceptible fungi. We did cytological studies on the mechanism of its fungicidal action against *Saccharomyces cerevisiae*. When cultures were treated with 5.0 μg of aureobasidin A per ml, the numbers of viable cells started to decrease after 2 to 3 h of incubation, and most cells had lost viability after 5 to 6 h. When cell death in the treated cultures began, amino acids released by the cells could be detected, indicating disruption of the cell membrane. The proportion of cells with a single small bud or two or more buds increased as the population of viable cells decreased. Most such cells had the DNA content of cells in the G_2 phase of the cell cycle, suggesting that the drug inhibited some cellular process involved in normal bud growth but did not affect DNA replication. Disruption of actin assembly was found in many cells treated for 2 to 3 h, as was chitin delocalization. The results suggest that aureobasidin A has a previously unknown mechanism of fungicidal action toward *S. cerevisiae*. It causes aberrant actin assembly, inhibiting the normal budding process and leading to cell death, probably through destruction of membrane integrity.

The incidence of life-threatening mycoses, particularly those caused by opportunistic fungi, appears to be increasing with growth in the number of immunocompromised patients, including those with AIDS, cancer, or organ transplants. Only a few systemic antifungal agents (amphotericin B, flucytosine, and several azole drugs including fluconazole, itraconazole, ketoconazole, and miconazole) are useful in the treatment of such mycoses, and none of these drugs is completely satisfactory in terms of efficacy, toxicity, antifungal spectrum, or the possibility of drug resistance. A polyene macrolide antibiotic, amphotericin B, is currently the "gold standard" for the treatment of deep-seated mycoses. Unlike drugs with a fungistatic action, amphotericin B is fungicidal toward both resting and growing cells of many pathogenic fungi. This antibiotic binds to membrane sterols and forms transmembrane pores, causing leakage of cell constituents and thus cell death (22). However, amphotericin B is toxic when administered intravenously. There is a need for safer and more effective antifungal agents. Many agents with fungicidal activity now under investigation are toxic to mammalian cells because of their many physiological similarities to fungal cells. New antifungal agents might be more useful if their mechanisms of action were different from those of agents being used now.

Aureobasidin A (AbA) (Fig. 1), an antifungal depsipeptide antibiotic produced by *Aureobasidium pullulans*, has strong fungicidal activity in vitro against many pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans*, and some *Aspergillus* spp. (11, 23, 24). AbA is effective given orally to mice with candidiasis, with little toxicity to the mice (24). This drug kills only growing cells of *C. albicans*; resting cells are unaffected (24). AbA does not inhibit the synthesis of DNA, RNA, or protein, nor does it decrease glucose incorporation into insoluble cell wall glucans of *C. albicans* (10). These results suggest that the action of AbA is different from the actions of amphotericin B and other antifungal agents and that AbA inhibits some intracellular process(es) essential for fungal growth. To identify the action of AbA, we studied its fungicidal effects on *Saccharomyces cerevisiae*, which has been widely used in cytologic and genetic studies. Here we report unusual cytologic features of *S. cerevisiae* cells treated with AbA, suggesting that the mechanism of action by which it kills yeasts and probably susceptible fungi is one not previously known.

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MATERIALS AND METHODS

Strain, medium, and culture conditions. Diploid cells of *S. cerevisiae* ATCC 9763 were maintained on YPG agar medium containing 1% yeast extract, 2% Polypeptone, 2% glucose, and 2% agar. Before use in experiments, the strain was cultured in YNBG medium containing 0.67% yeast nitrogen base (Difco, Detroit, Mich.) and 2% glucose at 25°C for 15 to 18 h. Cells in the exponential phase of growth (1×10^7 to 2×10^7 cells per ml) were harvested by centrifugation, washed with distilled water or saline, and used in the experiments that follow.

For measurement of the release of intracellular amino acids, yeast cells were cultured in YNBG medium free of amino acids (0.67% amino-acid-free yeast nitrogen base [Difco] and 2% glucose). AbA, more than 95% pure by high-pressure liquid chromatography, was prepared as described before (23) and used in the form of a methanol solution. Amphotericin B (Sigma, St. Louis, Mo.) was dissolved in dimethyl sulfoxide and diluted with methanol for use.

Measurement of cell viability. Cell viability was measured by an agar plate assay or by methylene blue staining (20). Some 10^5 cells were suspended in a tube containing 10 ml of fresh YNBG medium containing 5.0 μg of AbA per ml, 5.0 μg of amphotericin B per ml, or 1% methanol (control) and cultured at 25°C with shaking. Duplicate preparations were examined. For the agar plate assay, cell suspensions (100 μl) sampled from a culture 0, 1, 4, 8, and 24 h after incubation began were washed and diluted with saline. A 100-μl portion of the diluted cell suspension was spread on YPG agar plates containing 1.0% yeast extract, 2.0% Polypeptone, 2.0% glucose, and 2.0% agar. Duplicate preparations were examined. After culture for 2 or 3 days at 25°C, CFU were counted and used as the number of viable cells. For the assay with methylene blue, every hour from 0 to 6 h of incubation, 100 μl of the culture was sampled and mixed with an equal volume of methylene blue (Sigma) diluted (0.2 mg/ml in 0.1 M phosphate buffer [pH 4.6]). The mixture was left for 5 min at room temperature, and the total cells and stained (dead) cells in duplicate preparations were counted under a microscope. The viability was expressed as the mean of the percentage of unstained cells.

Measurement of amino acid release. Duplicate cultures of yeast cells in a flask containing 100 ml of YNBG medium free of amino acids at a concentration of 10^8 cells per ml were treated with 5.0 μg of AbA per ml, 5.0 μg of amphotericin B per ml, or 1% methanol during incubation at 25°C. Every hour from 0 to 6 h of incubation, 1 ml of the culture was sampled and centrifuged to remove cells. To the supernatant, 1 ml of 3.0% sulfosalicylic acid was added, and the mixture

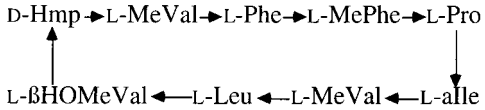

FIG. 1. Structure of AbA. Abbreviations: D-Hmp, 2(*R*)-hydroxy-3(*R*)-methylpentanoic acid; L-MeVal, *N*-methyl-L-valine; L-MePhe, *N*-methyl-L-phenylalanine; L-aIle, L-*allo*-isoleucine; L-βHOMeVal, β-hydroxy-*N*-methyl-L-valine.

was filtered through a membrane filter with 0.45-μm pores. Then the filtrate was freeze-dried, dissolved in 100 μl of 0.02 N HCl, and analyzed by an amino acid analyzer (L-8500; Hitachi, Tokyo, Japan) for glutamic acid and arginine. The lower limit of detection was 0.3 nmol of amino acids per ml. The amount of amino acids was expressed as the percentage of the amount when measured at 6 h.

Measurement of the amount of DNA by flow cytometry. Duplicate suspensions of yeast cells in a flask containing 100 ml of fresh YNBG medium at a concentration of 1×10^7 to 2×10^7 cells per ml were incubated with 5.0 μg of AbA per ml or without AbA. Portions of 1 ml were sampled at different times, washed twice with distilled water, and suspended in 10 ml of 70% ethyl alcohol. The suspension was kept for 15 to 18 h at 4°C, and then cells were collected by centrifugation, washed twice with 5 ml of 50 mM sodium phosphate buffer (pH 7.0), suspended in 1 ml of the same buffer containing 100 μg of RNase per ml (Takara Shuzo, Shiga, Japan), and kept at 37°C for 4 h. Then the cells were washed twice and transferred to 1 ml of sodium phosphate buffer containing 10 μg of propidium iodide per ml (Sigma). After 10 min of incubation at room temperature, the cells were recovered by centrifugation, washed twice, and suspended in 1 ml of sodium phosphate buffer. The DNA content of samples of 10^4 cells was examined by flow cytometry (Ortho Cytoron; Ortho Diagnostic Systems, Tokyo, Japan).

Photomicroscopic studies of yeast cells. The morphology of yeast cells incubated with 5.0 μg of AbA per ml or without AbA for 0, 2, 4, and 6 h was observed under a Nomarski microscope (NTF; Nikon, Tokyo, Japan) and photographed with an HFX-II camera (Nikon). For the categorization of cells according to their budding pattern, at least 500 cells were observed and classified into the five following patterns: A, unbudded cells; B, cells with a single small bud (length from interface with mother cell to furthest point, less than one-third the length of the mother cell on this axis); C, cells with a single medium-sized bud (length from one-third to two-thirds that of the mother cell); D, cells with a single large bud (length more than two-thirds that of the mother cell); and E, cells with two or more buds.

For fluorescence staining, yeast cells were sampled from cultures incubated with 5.0 μg of AbA per ml or without AbA for 0, 2, 3, and 6 h; fixed with 3.7% formaldehyde; and stained for actin or chitin with 0.33 μM phalloidin conjugated with rhodamine (Molecular Probes, Eugene, Oreg.) or 1.0 mg of Calcofluor white M2R per ml (Sigma), respectively, as described by Pringle et al. (19). Cells stained with one of these fluorochromes were observed under a photomicroscope (BH-2; Olympus, Tokyo, Japan) and photographed with Kodak Tmax 400 film (Kodak, Rochester, N.Y.).

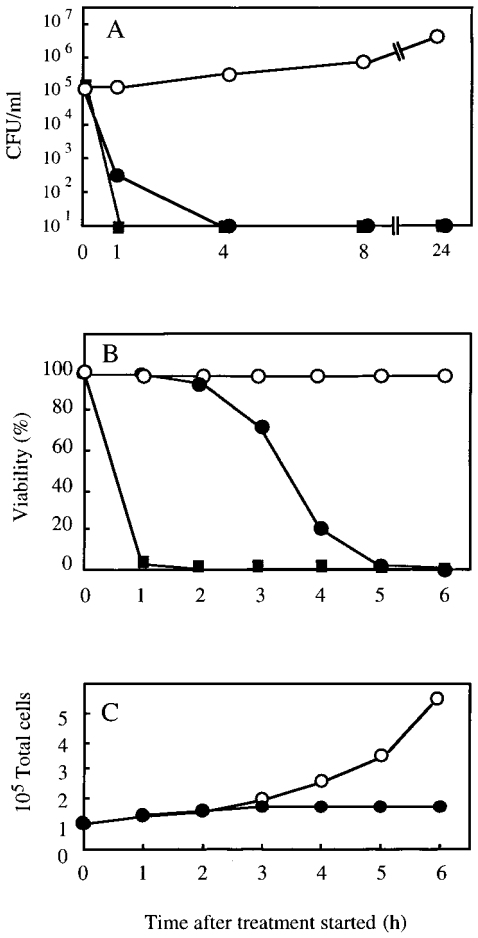

FIG. 2. Time-dependent killing effects of AbA and amphotericin B. Cells were suspended in fresh YNBG medium containing 5.0 μg of AbA per ml (●), 5.0 μg of amphotericin B per ml (■), or 1% methanol (○) and incubated at 25°C. (A) Portions were sampled at 0, 1, 4, 8, and 24 h, and CFU were counted. (B and C) Portions were sampled every hour; cell viability (B) was calculated, and the total number of cells (C) was counted.

RESULTS

Effects of drugs on cell viability. AbA killed growing cells; fewer than 1% of cells were viable when assayed on agar plates after 1 h of treatment, and more died later (Fig. 2A). This decrease in viability was slower than with amphotericin B at the range of concentrations tried. The lowest concentration of AbA and amphotericin B that inhibited the growth of exponentially growing cells was 2.5 and 0.63 μg/ml, respectively, in the assay with methylene blue. With 5.0 μg of AbA per ml, nonviable cells were found first at 2 h of incubation, and their numbers increased with time, reaching more than 98% by 6 h (Fig. 2B). Most cells incubated with amphotericin B were stained with methylene blue within 1 h. The total cell count of the culture incubated with AbA increased at the same rate as the control culture up to 2 h and stopped at 160% of the initial cell number (Fig. 2C). These curves were reproducible.

Disruption of cell membranes. During microscopic inspection of cultures treated with AbA, we found that vacuoles in the cells were stained with methylene blue after 2 to 3 h of treatment and that many vacuoles had been crushed at the time of cell death, indicating that the integrity of the cell membrane was lost.

We examined whether AbA affects the permeability barrier of the cell membrane by measuring the rate of release of glutamic acid and arginine, which are found mostly in the cytoplasm and vacuoles, respectively (12). With AbA, these amino acids were not detected in medium sampled after 2 h of incubation, but they were released later (Fig. 3). Alanine, proline, and threonine (in the cytoplasm) and lysine (in the vacuoles) were released as well (data not shown). The reference drug amphotericin B caused leakage of both glutamic acid and arginine when the medium was assayed 1 h after the start of incubation.

Changes in the morphology of cells after AbA treatment. By Nomarski microscopy, at 6 h after incubation started, untreated control cultures were seen to have only unbudded cells and cells with a single bud of various sizes (Fig. 4). Most cells treated with AbA had a single small bud or several buds at that time.

Figure 5 shows changes with time in the proportions of the different budding patterns in cultures with or without AbA. Control cultures contained no cells with budding pattern E, and the various proportions changed little with time. In cultures treated with AbA, the percentage of cells of patterns B and E increased with time and that of cells of pattern D decreased.

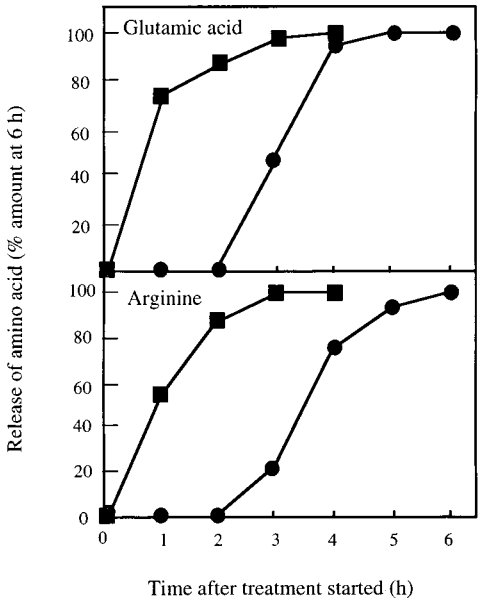

FIG. 3. Release of amino acids by cells treated with AbA or amphotericin B. YNBG medium containing 5.0 μg of AbA per ml (●), 5.0 μg of amphotericin B per ml (■), or 1% methanol was inoculated with cells, and the culture was incubated at 25°C. Portions were sampled every hour, and glutamic acid and arginine that had leaked into the medium from the cells were assayed. With amphotericin B, at 6 h, the amount of glutamic acid was 17.4 nmol/ml and that of arginine was 18.2 nmol/ml. With AbA, at that time these amounts were 9.0 and 31.0 nmol/ml, respectively. These amino acids leaked little from cells treated with 1% methanol (not shown).

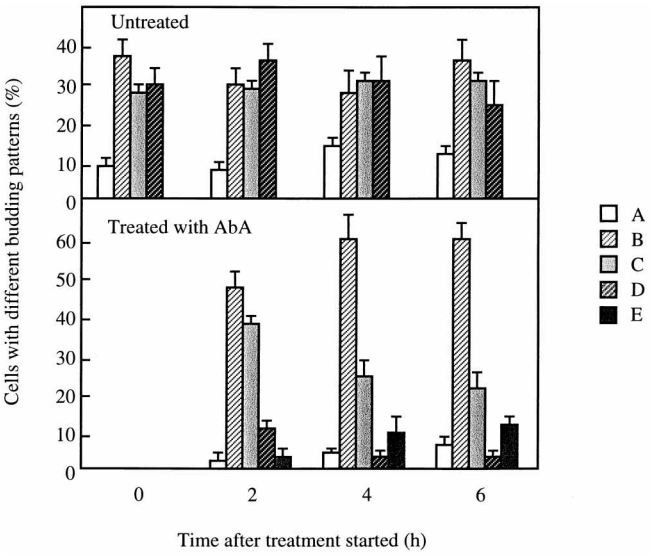

FIG. 5. Changes with time in proportions of various budding patterns in cultures with AbA. Cells were cultured with 5.0 μg of AbA per ml or without AbA, and samples were taken from each culture at the times shown. The cells were classified by their budding pattern under a photomicroscope as follows: A, unbudded cells; B, cells with a single small bud; C, cells with a single medium-sized bud; D, cells with a single large bud; and E, cells with two or more buds. Values are means + standard deviations of three experiments.

Accumulation of cells with high DNA content. The flow cytometric profiles of untreated control cultures showed that they had about 33% of cells with the DNA content of the G_1 phase and 67% of cells with the DNA content of the G_2 phase, which proportions changed little during the 6 h of incubation (Fig. 6). In cultures treated with AbA, the proportions were first seen to change 3 h after the start of incubation; the proportion of cells with the DNA content of the G_2 phase increased, accounting for 80 to 85% of all cells at 6 h. There were no cells with a DNA content higher than that in the G_2 phase.

Abnormalities caused by AbA in actin assembly and chitin deposition. Actin of control cells with a small bud was arranged asymmetrically with a few cortical patches at the growth site and filamentous cables extending to the growing bud (Fig. 7A). Chitin was deposited at the neck of budding cells and remained on the surface of the mother cells as a bud scar after cell division (Fig. 7B).

Most budding cells of cultures treated with AbA for 2 to 3 h had many cortical patches distributed or dispersed on the mother cells, and they lacked filamentous actin cables (Fig. 7C and D). Some abnormal cells had two depositions of cortical patches of actin: one was at the bud and the other was at the prebud site(s) on the mother cell (Fig. 7D, arrow). At the same time, abnormal patch-like depositions of materials stained with Calcofluor were observed on the cell surface of many cells treated with AbA, especially on premature buds (Fig. 7E and F).

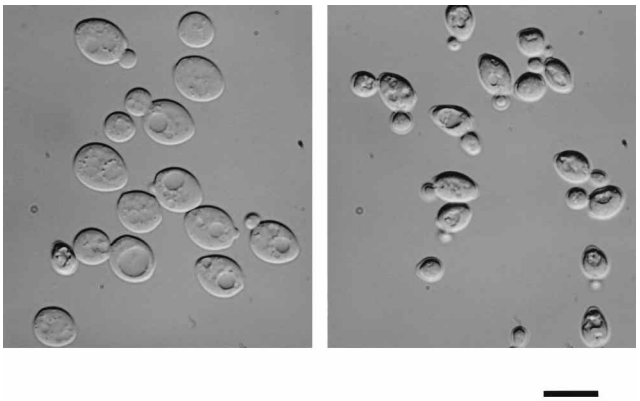

FIG. 4. Nomarski photomicrographs of yeast cells from an untreated culture (left) and from a culture treated with 5.0 μg of AbA per ml (right) for 6 h. Bar, 20 μm.

DISCUSSION

The viability of most cells treated with AbA at a time when amphotericin B was already killing cells and the later failure of cells treated with AbA to form colonies suggested that the fungicidal action of AbA, unlike that of amphotericin B, is associated with cell growth. In growing cells, DNA replication and bud growth occur at the same time, followed by cell division. Our results showed that AbA inhibited bud growth, after which the number of cells with a premature bud(s) increased and DNA replication continued. This imbalance in the cell growth increased the number of cells with the DNA content of the G_2 phase. Normally, cells with this DNA content have one medium-sized to large bud, not premature buds. These results suggested that the inhibition of cell growth by AbA is due primarily to inhibition of bud growth, not DNA replication.

AbA also caused the release of intracellular amino acids. Such findings have been reported for temperature-sensitive *S. cerevisiae* mutants with the *pkc1* gene (14) or several *ras* superfamily genes such as *rho1*, *rho3*, and *rho4* (16, 25). These genes are involved in bud growth and required to confer osmotic resistance on a newly formed cell by stimulating surface

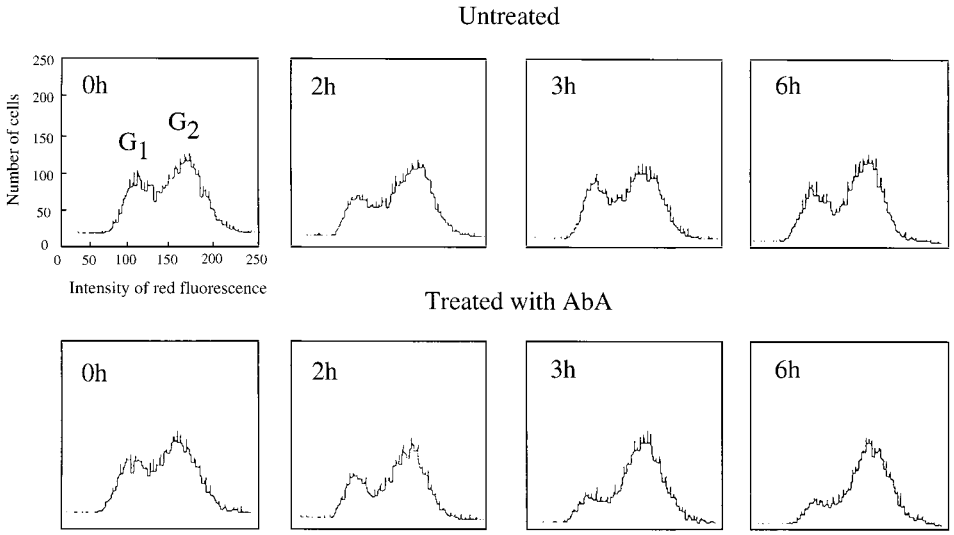

FIG. 6. Effects of AbA on DNA replication. Cells were cultured with 5.0 μg of AbA per ml or without AbA. Portions were sampled from each culture, fixed with ethyl alcohol, and stained with propidium iodide. In control culture, the proportions of cells with the DNA content of the G_1 phase were 38, 34, 36, and 33% at 0, 2, 3, and 6 h, respectively. The proportions in culture treated with AbA were 37, 32, 19, and 19% at 0, 2, 3, and 6 h, respectively. In another experiment, the proportions of cells with the DNA content of the G_1 phase at 6 h were 34% in control culture and 15% in culture treated with AbA. *y* axis, number of cells; *x* axis, intensity of red fluorescence.

growth at the bud site. The lethality of these mutants under a nonpermissive condition is suppressed by the addition of 100 mM $CaCl_2$ or osmotic stabilizers such as 1 M sorbitol. The killing effect of AbA was not suppressed by the addition of 100 mM $CaCl_2$, 1 M sorbitol, or 0.5 M KCl (data not shown), suggesting that the action of AbA is not directly associated with the functions of the products of these genes and may inhibit some other mechanism(s) involved in the organization of bud growth and maintenance of the integrity of the cell membrane.

The relationship between the bud growth and the polarized distribution of the cytoskeletal constituents actin and chitin is close (4–6). Phenotypic analysis of conditionally lethal actin mutants has shown that actin assembly is involved in the deposition of chitin and in the movement of secretory vesicles to the cell surface (18). Cells with type B or C budding patterns, which accumulated after the treatment with AbA, should have both kinds of actin, cortical patches at growing buds and distinct filamentous cables extending to the buds, and also chitin at bud necks and scars. However, almost no cells treated with AbA had actin cables, and their cortical patches were not in a polarized arrangement. Furthermore, cells treated with AbA had delocalized chitin in patches on the cell surface of premature buds. These observations indicate that AbA inhibited normal actin assembly, especially the formation of filamentous cables, resulting in mistargeting of materials like chitin for new cell walls of growing buds. Disarranged actin and diffuse chitin have been observed in a variety of cell division cycle mutants including *cdc24* (21), *cdc42*, *cdc43* (1), *cap1*, *cap2* (3), *pfy1* (8), *sac6* (2), *cof1* (17), and *tpm1* (15). These mutants have disordered or dispersed chitin on the cell surface, unlike the patch-like deposition of cells treated with AbA. Cytochalasin A, an inhibitor of actin assembly, causes abnormal distribution of actin patches and delocalization of chitin (Calcofluor-binding cell wall substances) in yeast-form cells of the dimorphic yeast *Candida tropicalis*, and multibudding cells with some large buds become numerous after its treatment (13). These abnormalities partly resemble those caused in *S. cerevisiae* by AbA treatment but differ in the kind of abnormal actin and in the size and the number of buds of the multibudding cells. AbA is less toxic to mammalian cells than cytochalasin A (data not shown), suggesting that AbA does not act on the actin assembly of mammalian cells and differs from known actin inhibitors such as cytochalasins.

Some cells with a premature bud had one deposition of cortical actin patches on the bud and another deposition at a prebud site on the mother cell, so cells treated with AbA when they already had one premature bud were about to form a second bud at the prebud site on the mother cell. This phenomenon is consistent with the great increase of type E cells (with two or more premature buds). These results suggest that AbA does not affect the actin assembly that is essential for bud formation but rather inhibits actin assembly that is needed for formation of filamentous actin cables for bud growth and also inhibits the transport of materials needed for bud growth.

Our group and Heidler and Radding isolated an essential gene conferring resistance to AbA by mutation, and the gene was designated *AUR1* (aureobasidin resistance gene) (9, 10). A transformant in which expression of Aur1p is controlled by the GAL1 promoter has abnormal deposition of chitin and plasmolysis of the cytoplasm from the cell wall in a galactose-free medium, as do cells treated with AbA. However, filamentous actin cables are formed normally in the Aur1p-depleted cells. This discrepancy may be caused by the much slower death of Aur1p-depleted cells than of cells treated with AbA. Over 50% of the transformant cells survive 12 h after the shift to the Aur1p depletion condition, whereas AbA caused rapid death between 3 and 5 h after its addition, and there were no cells alive at 5 h. The relationship between actin assembly and Aur1p is not known, but knowledge of the action of AbA and the function of Aur1p should provide clues to clarify the mechanism of cell growth, especially bud growth, of *S. cerevisiae*.

Greene et al. (7) have reported the effects of an AbA analog, SCH56301, on *C. albicans* and *S. cerevisiae*, showing aberrant bud growth in both organisms and disorganized cell wall materials in *C. albicans*. Thus, the abnormal accumulation of materials stained with Calcofluor after AbA treatment occurs in both of these yeasts, suggesting that the fungicidal mecha-

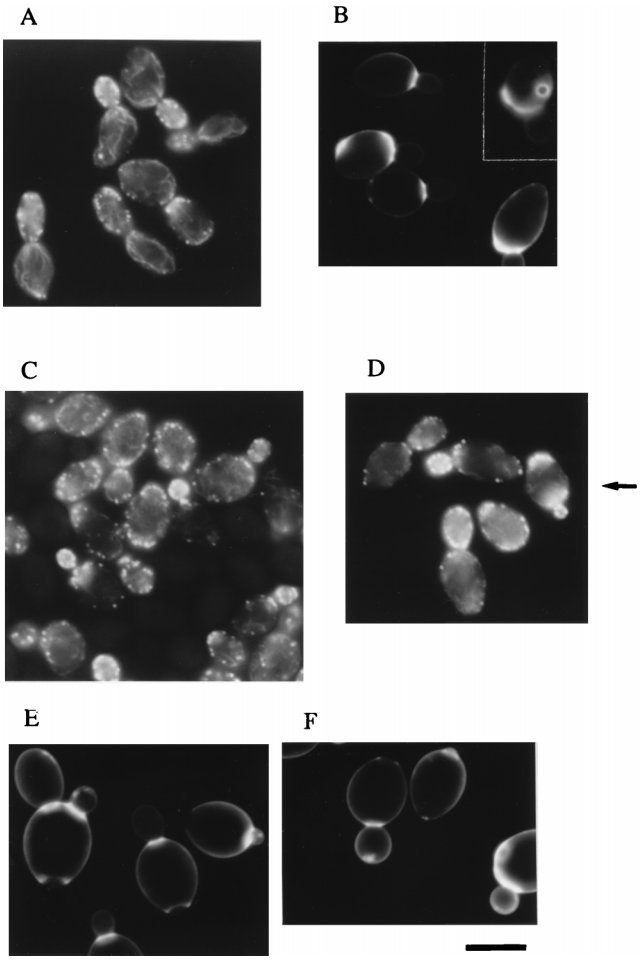

FIG. 7. Abnormalities caused by AbA in actin assembly and chitin deposition. Cells cultured for 3 h with 5.0 μg of AbA per ml or without AbA were sampled, and actin and chitin were stained as described in Materials and Methods. (A, C, and D) Actin assembly stained with rhodamine-phalloidin. (B, E, and F) Chitin deposition stained with Calcofluor. (A and B) Control cells; (C, D, E, and F) cells treated with AbA. The arrow indicates cells with two depositions of cortical patches of actin. Bar, 10 μm.

nism of AbA in the two is the same. The effects of AbA on yeasts may indicate its selective toxicity. Identification of the mechanism may facilitate screening for fungicidal antibiotics with high selective toxicity.

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